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Gene Expression of Hematoregulatory Cytokines Is Elevated Endogenously After Sublethal Gamma Irradiation and Is Differentially Enhanced by Therapeutic Administration of Biologic Response Modifiers[1]

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Prompt, cytokine-mediated restoration of hematopoiesis is a prerequisite for survival after irradiation. Therapy with biologic response modifiers (BRMs), such as LPS, 3D monophosphoryl lipid A (MPL), and synthetic trehalose dicorynomycolate (S-TDCM) presumably accelerates hematopoietic recovery after irradiation by enhancing expression of cytokines. However, the kinetics of the cytokine gene response to BRMs and/or irradiation are poorly defined. One hour after sublethal (7.0 Gy) ^{60}Cobalt gamma irradiation, B6D2F1/J female mice received a single i.p. injection of LPS, MPL, S-TDCM, an extract from *Serratia marcescens* (Sm-BRM), or Tween 80 in saline (TS). Five hours later, a quantitative reverse transcription-PCR assay demonstrated marked splenic gene expression for IL-1β, IL-3, IL-6, and granulocyte-CSF (G-CSF). Enhanced gene expression for TNF-α, macrophage-CSF (M-CSF), and stem cell factor (SCF) was not detected. Injection of any BRM further enhanced cytokine gene expression and plasma levels of CSF activity within 24 h after irradiation and hastened bone marrow recovery. Mice injected with S-TDCM or Sm-BRM sustained expression of the IL-6 gene for at least 24 h after irradiation. Sm-BRM-treated mice exhibited greater gene expression for IL-1β, IL-3, TNF-α, and G-CSF at day 1 than any other BRM. When challenged with 2 $LD_{50/30}$ of *Klebsiella pneumoniae* 4 days after irradiation, 100% of Sm-BRM-treated mice and 70% of S-TDCM-treated mice survived, whereas $\leq$30% of mice treated with LPS, MPL, or TS survived. Thus, sublethal irradiation induces transient, splenic cytokine gene expression that can be differentially amplified and prolonged by BRMs. BRMs that sustained and/or enhanced irradiation-induced expression of specific cytokine genes improved survival after experimental infection. *The Journal of Immunology*, 1994, 153: 2321.

Bone marrow failure is an inevitable consequence of life-threatening radiation injuries, and the severity and duration of the resulting neutropenia influence morbidity and mortality (1, 2). Survival after irradiation requires regeneration of bone marrow from quiescent hematopoietic stem cells (3, 4). Proliferation and differentiation of these hematopoietic progenitors depends on the endogenous expression of hematoregulatory cytokines (3–5). Therapeutic administration of IL-1, TNF-α, IL-6, or SCF[3] after lethal irradiation improves survival in animals, suggesting the importance of these hematopoietic regulatory cytokines after radiation (6–10). Neta et al. (11–14) report that pretreatment of irradiated mice with Abs specific for IL-1 receptor, TNF-α, IL-6, or SCF significantly decreases survival, whereas administration of

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[3] Abbreviations used in this paper: SCF, stem cell factor; AFRRI, Armed Forces Radiobiology Research Institute; BRM, biologic response modifier; MPL, 3D monophosphoryl lipid A; S-TDCM, synthetic trehalose dicorynomycolate; Sm-BRM, extract from *Serratia marcescens*; TS, 2% Tween 80 in 0.9% NaCl; RT-PCR, reverse transcription PCR; HPRT, hypoxanthine guanine phosphoribosyl transferase; CFU-GM, CFU granulocyte-macrophage; pluripotential myeloid progenitor; CFU-S, CFU spleen; multipotential progenitor; G-CSF, granulocyte-CSF; M-CSF, macrophage-CSF.

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Abs specific for IL-3, IL-4, GM-CSF, or IFN-γ does not alter survival. These data suggest that after radiation injury presence of specific cytokines are critical for restoration of bone marrow and host survival but do not address the issue of induction or elevation of these cytokines.

It is clear that the therapeutic administration of a single hematoregulatory cytokine can accelerate hematopoietic recovery in vivo after radiation injury. However, in vitro studies indicate that bone marrow must be exposed to multiple hematopoietic growth factors to achieve optimal myeloproliferation (3, 4). An alternative to the therapeutic administration of multiple cytokines in vivo after irradiation is the use of a single BRM that induces the endogenous expression of many hematoregulatory cytokines (15). For example, LPS stimulates expression of several CSFs, IL-1, TNF-α, and IL-6, and administration of LPS to mice after lethal irradiation improves survival (16–18). Unfortunately, preclinical and clinical studies indicate that LPS is prohibitively toxic (19–22). Consequently, other bacterially derived BRMs have been developed that retain radioprotective and/or radiotherapeutic properties but exhibit less toxicity. These agents include MPL, a dephosphorylated derivative of the lipid A moiety of LPS; S-TDCM, a chemical preparation similar to a native cell wall-associated glycolipid isolated from *Corynebacterium* species; and Sm-BRM, a cell membrane/ribosomal RNA-sized vesicle preparation isolated from *Serratia marcescens*.

Although therapeutic administration of BRMs appears to improve host survival by enhancing endogenous expression of cytokines that accelerate bone marrow recovery, the effects of BRMs on the kinetics of cytokine gene expression after irradiation are not well defined, largely because quantitation of cytokine gene expression in hypoplastic, irradiated tissues has been technically difficult (17, 23). Recently developed RT-PCR assay techniques, however, enable quantitation of gene expression of multiple cytokines from limited quantities of mRNA (24, 25). The purpose of this investigation was to determine in a murine model of sublethal gamma irradiation whether: 1) irradiation alone is sufficient to induce cytokine gene expression; 2) therapeutic administration of BRMs alters post-irradiation cytokine gene expression; and 3) therapeutic administration of BRMs alters hematopoietic recovery and/or improves survival after experimental bacterial infection. Our results indicate that gene expression of specific cytokines is induced within 5 h after gamma irradiation and is differentially enhanced and/or prolonged by the therapeutic administration of BRMs. Despite the observation that therapeutic administration of any one of these BRMs accelerated bone marrow recovery, only the administration of S-TDCM or Sm-BRM sustained expression of specific, irradiation-induced cytokine genes and improved survival of irradiated mice infected with *Klebsiella pneumoniae*. These data then suggest that the selection of BRMs capable of enhancing and/or prolonging gene expression of hematoregulatory cytokines is an important factor in determining survival to infection after sublethal irradiation.

Materials and Methods

Mice

B6D2F1 J female mice (The Jackson Laboratory, Bar Harbor, ME), 11 wk of age, 18 to 22 g, were held in quarantine for 2 wk. Necropsy specimens from sentinel mice were examined to ensure the absence of specific intestinal bacteria and common murine diseases by microbiology, serology, and histopathology. Up to 10 mice were housed in sanitized 46 cm × 24 cm × 15 cm polycarbonate boxes with a filter cover (Microisolator, Lab Products, Inc., Maywood, NJ) on sterilized hardwood chip bedding in a facility accredited by the American Association for Accreditation of Laboratory Animal Care. Mice were given feed and acidified (pH 2.5) water freely. The animal holding room was maintained with conditioned fresh air that was changed at least 10 times per hour at approximately 21°C and 50% (± 10%) relative humidity and with a 12-h light/dark full spectrum lighting cycle. All research was approved by the Institutional Animal Care and Use Committee.

Irradiation

Mice were placed in ventilated Plexiglas containers and exposed bilaterally to gamma radiation from the AFRRI ^{60}Co source, as previously described (26). Exposure time was adjusted so that each animal received a 7.0-Gy midline tissue-absorbed dose at a nominal dose rate of 0.4 Gy/min at ambient temperature. Using a standardized technique, the midline tissue dose rate was measured by placing a 0.5-cc tissue equivalent ionization chamber at the center of a 2.5-cm diameter cylindrical acrylic mouse phantom before irradiation of animals (27). The tissue-to-air ratio, defined as the ratio of the dose rate in free air to the dose rate measured in the phantom, for this array was 0.96. Variation within the exposure field was less than 3% (26). The lethal dose for 50% of B6D2F1 J female mice 30 days after exposure ($LD_{50/30}$) to this source was 9.6 ± 0.30 Gy.

BRMs

All BRMs were administered i.p. 1 h after irradiation as sterile solutions at dosages indicated in the results section. S-TDCM (Ribi ImmunoChem Research, Inc., Hamilton, MT) was prepared as an aqueous suspension by a modification of the procedure of Vosika and Gray (28), as described previously (23). Briefly, 2-mg aliquots of S-TDCM were solubilized in 0.2 ml of chloroform methanol (9:1), placed in a 5-ml Potter-Elvehjem tissue homogenizer, dried under nitrogen, and suspended with homogenization in TS to contain 400 μg/ml. Synthesis of S-TDCM was previously described (23, 29). Sm-BRM (generously provided by Dr. Catherine McCall, Cell Technology, Inc., Boulder, CO) contains two distinct particle classes, i.e., natural bacterial cell membrane vesicles and ribosomes derived from *Serratia marcescens* (31). Sterile distilled water was used to dilute 1000 μg of lyophilized Sm-BRM to a final concentration of 200 μg/ml. Sm-BRM was injected within 1 h of reconstitution. MPL (400 μg/ml) derived from *Salmonella enteritidis* ser. *typhimurium* (Ribi ImmunoChem Research, Inc.) and protein-free, phenol water-extracted LPS (50 μg/ml) from *Escherichia coli* K235 (kindly provided by Dr. Stephanie N. Vogel, Uniformed Services University of the Health Sciences, Bethesda, MD) were prepared as described previously (32).

Collection of tissues

Sterile technique was used throughout. Mice were deeply anesthesized with methoxyflurane (Pitman-Moore, Washington Crossing, NJ) before exsanguination by percutaneous cardiac puncture using a syringe rinsed with sodium heparin solution. Blood was transferred into a vial that contained ethylene diamine tetraacetate then placed immediately on ice and centrifuged within 1 h at 4°C. Separated plasma was then frozen at −70°C until assayed for CSF activity. Spleens were pressed through stainless steel mesh into 5 ml RPMI 1640 medium to disperse cells. Bone marrow cells were obtained by flushing both tibia with 1 ml of RPMI 1640 per tibia. A 500-μl aliquot of both cell suspensions was monodispersed by gentle pipetting, centrifuged, resuspended in RPMI 1640 supplemented with 10% FCS, and kept on ice until cultured for CFU-GM.

Table I. *Primer sequences for amplification of cytokine cDNA during PCR and probe sequences for detection of amplified DNA product on Southern blot*

Cytokine	Antisense and Sense Primer	Bases Spanned (size)	Probe	Bases Spanned	No. of PCR Cycles	Ref.
HPRT	GTTGGATACAGGCCAGACTTTGTTG GATTCAACTTGCGCTCATCTTAGGC	514–538 652–678 (164)	GTTGTTGGATATGCCCTTGAC	562–582	12	24
G-CSF	GAGCAAGTGAGGAAGATCCAG TTAGGCACTGTGTCTGCTGC	572–592 1072–78/ 1325–37 (172)	AGTTGTGTGCCACCTACA	618–22/ 971–83	25	63
M-CSF	ATTGGGAATGGACACCTGAAG GCTGTTGTTGCAGTTCTTGG	286–306 662–681 (396)	TTCCATGAGACTCCTCTC	571–588	21	64
IL-3	ACTGATGATGAAGGACC TTAGCACTGTCTCCAGATC	1000–1016 2485–2504 (256)	TCGGAGAGTAAACCTGTCCA	2030–2049	28	65
TNF-α	TTCTGTCTACTGAACTTCGGGG ACTTGGGCAGATTGACCTCAGC	955–976 2437–2458 (514)	CCCGACTACGTGCTCCTCACC	2248–2268	23	65
IL-6	TTCCATCCAGTTGCCTTCTTGG CTTCATGTACTCCAGGTAG	73–94 414–432 (359)	ACTTCACAAGTCCGGAGA	127–144	21	24
IL-1β	GGGATGATGATGATAACCTG TTGTCGTTGCTTGGTTCTCCT	397–416 572–592 (195)	GGCTCCGAGATGAACAACAA	454–473	14	66
SCF (c-*kit* ligand)	GATAACCCTCAACTATGTCGC TACTGCTGTCATTCCTAAGG	156–176 576–595 (439)	GAGGCCAGAAACTAGATCCT	384–403	25	67

The remainder of the splenocytes were promptly centrifuged, resuspended in 200 μl HBSS, homogenized 3 to 5 s in 1.5 ml RNAzol B (Cinna/Biotecx Laboratories International, Inc., Friendswood, TX) by using a tissue homogenizer, frozen in liquid nitrogen, and stored at −70°C until RNA extraction could be performed.

Culture of granulocyte-macrophage progenitor cells

Myeloid progenitors (CFU-GM) in the bone marrow and spleen were quantitated in soft agar using modifications of a previously described technique (31). Briefly, 1×10^5 nucleated bone marrow cells or 1×10^6 splenocytes were cultured in 1 ml of 0.3% agar-McCoy's 5a medium supplemented with 15% FCS and 100 μl of an Amicon-filtered human urine concentrate that served as a source of CSF. Cells were plated in 35-mm plastic culture dishes and incubated for 7 days in 6% CO_2 at 37°C. Colonies comprised of ≥50 cells were enumerated with a dissecting microscope, and data were expressed as CFU-GM per organ.

Bioassay for CSF in plasma

The presence of CSF activity in plasma was detected with modifications of a bioassay described previously (31). Briefly, twofold dilutions of pooled plasma were added to duplicate 35-mm tissue culture plates (final plasma concentrations were 2.5, 5, and 10%). Plasma samples were then covered with 1 ml of 0.3% agar in McCoy's 5a culture medium supplemented with 15% FCS that contained 1×10^5 nucleated tibial bone marrow cells harvested from nonirradiated B6D2F1/J mice. Cultures were incubated in 6% CO_2 at 37°C for 7 days and colonies were counted with a dissecting microscope. CSF activity, expressed as U/ml, was calculated as the product of the reciprocal of the sample dilution and the mean number of colonies per plate.

RT-PCR

RNase-free plastic ware, water, and surgical gloves were used throughout the procedure.

Isolation and purification of RNA. Total RNA was isolated using modifications of a one-step phenol guanidium isothiocyanate chloroform extraction technique (33). After 2-propanol precipitation and UV spectrophotometric quantitation for total cellular RNA, 5 μg of RNA was electrophoresed in a 2% formaldehyde agarose gel that contained ethidium bromide to ascertain whether the RNA was undegraded and accurately quantitated. With this technique, the final preparation was free of DNA and protein, with a 260:280-nm ratio >1.8

RT reaction. RT of RNA was performed in a final volume of 25 μl using the technique described by Svetic et al. (24), which is a modification of that described by Diamond et al. (34). The final mixture was heated to 70°C for 5 min to denature the RNA and cooled on ice. Then, 1.2 μl of RT (200 U/ml) was added to the mixture, centrifuged to bring down any condensation, and incubated at 37°C for 60 min. The reaction was then heated at 90°C for 5 min then quickly chilled on ice.

PCR. Sense and antisense primers, specific for the desired cytokine, were added to the RT reaction product, along with PCR buffer, deoxynucleotide mixtures, and Taq polymerase (24). For each cytokine, the optimum number of cycles was determined by achieving a detectable concentration that was well below saturating conditions, as previously described (24). To further ensure that equal amounts of RNA were added to each PCR reaction, primers for a housekeeping gene, HPRT, were used to amplify the cDNA that was reverse transcribed from total RNA and probed in the same manner as the cytokines. Amplified product was identified by Southern blot analysis (35) and quantitated by densitometry with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA), as previously described (24). The probes were specifically selected to hybridize to a portion of the amplified segment that was between the nucleotide sequences complementary to the primers to ensure identification of the amplified segment. Sequences for primers and probes for IL-6 and HPRT were as previously described (24). Sequences for HPRT, G-CSF, M-CSF, IL-3, TNF-α, IL-6, IL-1β, and the c-*kit* ligand, SCF, appear in Table I.

Bacteria

K. pneumoniae AFRR1 7 was prepared as previously described (36). Bacteria were injected s.c. into mice that were lightly anesthetized with methoxyflurane.

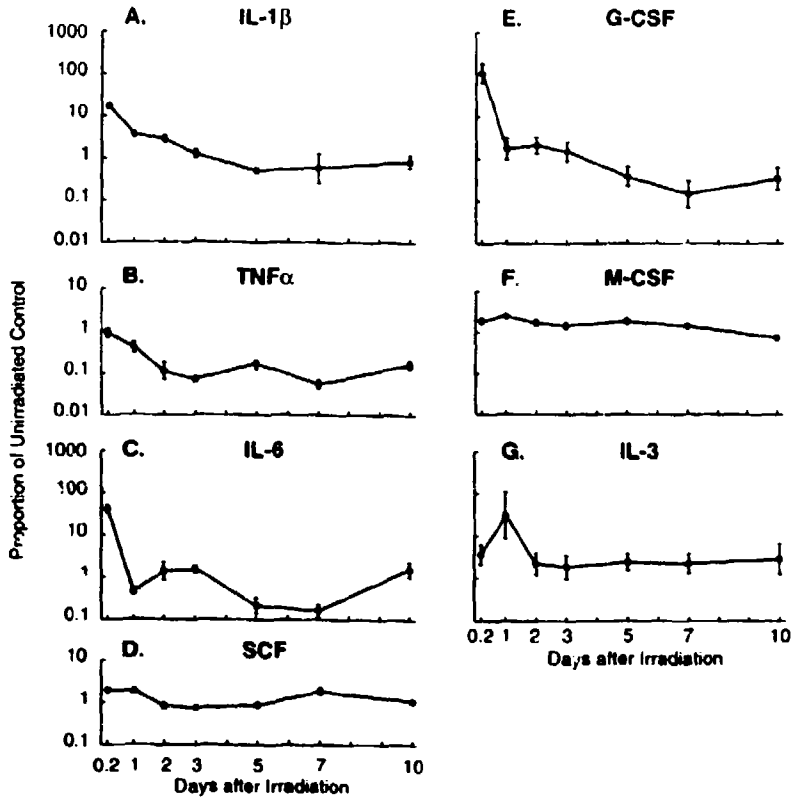

FIGURE 1. Effect of sublethal gamma-irradiation on kinetics of splenic cytokine gene expression determined by RT-PCR. One hour after gamma irradiation (7.0 Gy) groups of five mice received a single i.p. injection of TS and were killed at the times indicated. Splenic cytokine mRNA levels of IL-1β, IL-3, IL-6, TNF-α, G-CSF, M-CSF, and SCF are expressed as a proportional change relative to the respective cytokine mRNA levels in untreated, nonirradiated controls.

Survival measurement and statistical evaluation

Thirty-day survival among the various experimental groups of irradiated mice were compared with the generalized Savage (Mantel-Cox) procedure (Program 1L, BMD Statistical Software, Inc., Los Angeles, CA). When analyzing cytokine gene expression by RT-PCR, a log transform was made of the ratio of the corrected densities for cytokine mRNA measurements to the corrected densities for the housekeeping gene, HPRT. For each time period and treatment group (e.g., LPS, MPL, S-TDCM, Sm-BRM, and TS), the mean of these values was calculated and the mean of the log transformed ratio for untreated values was subtracted from these means. The antilog values of these differences were then plotted along with their standard error. A proportion of the nonirradiated control of "1" indicates that the value for a treated group equals the value for the untreated, unirradiated control group. In addition, a one-way analysis of variance was performed on the log transformed ratios at each time period to compare treatments. If the one-way analysis of variance was significant, then a Newman-Keul's test was conducted to determine where statistically significant differences among treatment groups existed. Unless stated otherwise, data are calculated as the mean ± SEM, and statistical significance is assumed for $p \leq 0.05$.

Results

Effects of irradiation on cytokine gene expression

Irradiation (i.e., irradiated mice injected with TS) was sufficient to induce an early (4 h after TS or 5 h after irradiation) increase in cytokine gene expression for IL-1β, IL-3, IL-6, and G-CSF but TNF-α, M-CSF, and SCF mRNA levels were unaltered compared with untreated, unirradiated control mice (Fig. 1). At 24 h after radiation, mRNA levels for all cytokines, except IL-3, had returned to baseline. Exposure to sublethal irradiation alone thus induces a transient expression of mRNA for IL-1β, IL-6, and G-CSF and a slightly more prolonged expression of mRNA encoding IL-3.

Effects of BRMs on cytokine gene expression after irradiation

The kinetics of cytokine gene expression of IL-1β, IL-3, IL-6, TNF-α, G-CSF, M-CSF, and SCF were monitored for 10 days beginning 4 h after a single i.p. injection of either 25 μg LPS, 200 μg MPL, 200 μg S-TDCM, 100 μg Sm-BRM, or 0.5 ml TS. Because the biologic responses were equivalent at the dosages of LPS and MPL used in this study, only data from LPS-injected mice are presented for cytokine gene expression after injection of these two BRMs. Initially, injection of BRMs tended to enhance and/or prolong cytokine gene expression but over time gene expression returned toward levels observed in irradiated mice injected with TS. For example, IL-1β mRNA levels 4 h after injection (i.e., 5 h after irradiation) were not increased by BRMs above the initial ~15-fold increase seen in TS-injected, irradiated controls (Fig. 2*A*), but injection of S-TDCM or Sm-BRM maintained IL-1β gene expression above levels seen in TS-injected mice at day 2. Only an Sm-BRM injection prolonged IL-1β gene expression beyond day 2.

TNF-α gene expression was modestly enhanced (~5-fold increase over irradiated controls) at 4 h after injection

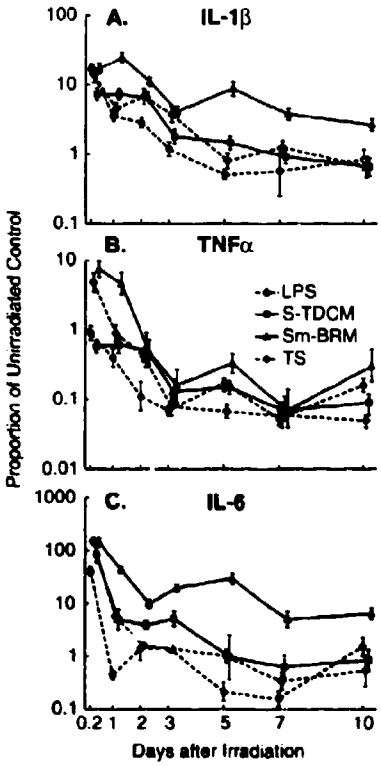

FIGURE 2. Comparative effects of a single i.p. injection of BRMs on gene expression for IL-1β, TNF-α, and IL-6 after sublethal gamma irradiation. One hour after irradiation, LPS, MPL, S-TDCM, Sm-BRM, or TS was injected into groups of five mice and splenic mRNA levels quantitated serially by RT-PCR for IL-1β (*A*), TNF-α (*B*), and IL-6 (*C*). Data are expressed as a proportional change relative to the respective cytokine mRNA levels in untreated, unirradiated controls. Cytokine gene expression for LPS and MPL are comparable and only data for LPS-injected mice are shown.

of LPS and at both 4 and 24 h after injection of Sm-BRM (Fig. 2*B*). S-TDCM administration failed to induce expression of TNF-α mRNA at any time point after injection. Although irradiation resulted in an ~40-fold increase in levels of IL-6 mRNA, injection of BRMs further enhanced IL-6 gene expression at 4 and 24 h after injection (Fig. 2*C*). Subsequently, only Sm-BRM or S-TDCM maintained levels of IL-6 gene expression above levels found in TS-injected, irradiated controls, and beyond day 3, only irradiated mice that received Sm-BRM continued to express high levels of IL-6 mRNA.

Levels of G-CSF mRNA were increased ~100-fold 4 h after injection of TS but subsequent determinations found G-CSF mRNA levels equal to or below those observed in unirradiated controls (Fig. 3*A*). Administration of LPS or Sm-BRM amplified gene expression threefold and fourfold, respectively, above levels seen in TS-treated mice at 4 h but S-TDCM injection did not enhance G-CSF gene expression. However, injection of LPS, S-TDCM, or Sm-

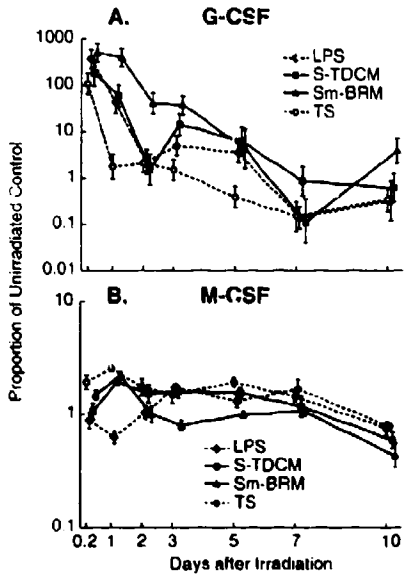

FIGURE 3. Comparative effects of a single i.p. injection of BRMs on gene expression for G-CSF and M-CSF after sublethal gamma irradiation. One hour after irradiation, LPS, MPL, S-TDCM, Sm-BRM, or TS was injected into groups of five mice and splenic mRNA levels quantitated serially by RT-PCR for G-CSF (*A*) and M-CSF (*B*). Data are expressed as a proportional change relative to the respective cytokine mRNA levels in untreated, unirradiated controls. Cytokine gene expression for LPS and MPL were comparable and only data for LPS-injected mice are shown.

BRM was associated with enhanced levels of G-CSF mRNA at days 1, 3, and 5 after irradiation, although not to the levels observed in the Sm-BRM treatment group. In contrast, BRM administration failed to alter splenic gene expression of M-CSF at any time point after irradiation (Fig. 3*B*).

IL-3 and SCF genes were also expressed differentially in response to administration of BRMs (Fig. 4). IL-3 gene expression was markedly enhanced at 4 h and at days 1, 2, and 5 after injection of Sm-BRM compared with irradiated mice injected with TS (Fig. 4*A*). LPS, MPL, and S-TDCM did not augment IL-3 gene expression, and, similar to the pattern of gene expression observed for M-CSF, splenic mRNA levels of SCF were not enhanced at any time after irradiation by any of the BRMs evaluated in this study (Fig. 4*B*).

Effects of BRMs on plasma CSF activity

Irradiated mice that were given saline exhibited a minimal increase in plasma CSF activity (110 ± 30 U/ml) 5 h after irradiation but all subsequent plasma determinations for CSF activity were comparable to plasma CSF levels in unirradiated, untreated controls (i.e., ≤40 U/ml) (Fig. 5). Administration of BRMs resulted in an enhanced but transient plasma CSF response 4 h after injection. The CSF

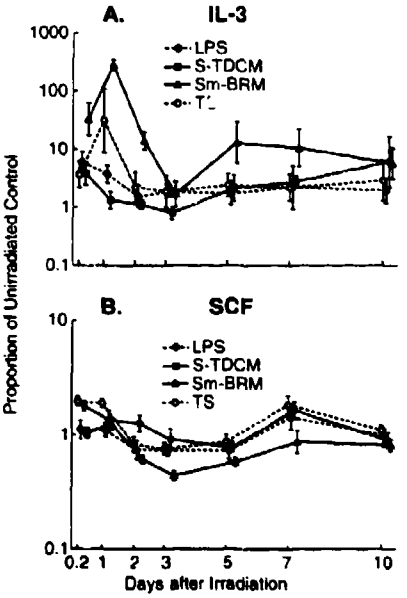

FIGURE 4. Comparative effects of a single i.p. injection of BRMs on gene expression of IL-3 and SCF after sublethal gamma irradiation. One hour after irradiation, LPS, MPL, S-TDCM, Sm-BRM, or TS was injected into groups of five mice and splenic mRNA levels quantitated serially by RT-PCR for IL-3 (*A*) and SCF (*B*). Data are expressed as a proportional change relative to the respective cytokine mRNA levels in untreated, unirradiated controls. Cytokine gene expression for LPS and MPL were comparable and only data for LPS-injected mice are shown.

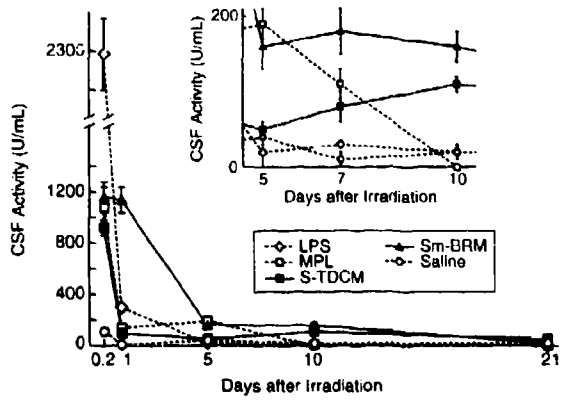

FIGURE 5. Comparative effects of BRMs on the plasma CSF response after sublethal gamma irradiation. One hour after irradiation, LPS, MPL, S-TDCM, Sm-BRM, or saline was injected into groups of three mice and plasma was collected at 4 h and on days 1, 2, 3, 5, 7, 10, 14 and 21 after irradiation. Serial dilutions of pooled plasma (three mice/pool) were assayed in triplicate for CSF activity, as outlined in the *Materials and Methods* section. Data represent one of two identical experiments with comparable results.

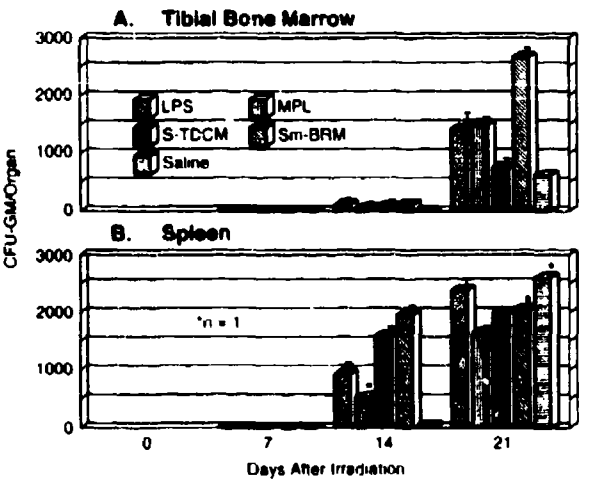

FIGURE 6. Comparative effects of BRMs on recovery of myeloid progenitors (CFU-GM) in bone marrow and spleen after sublethal gamma irradiation. One hour after irradiation, LPS, MPL, S-TDCM, Sm-BRM or saline was injected into groups of three mice and animals were killed at the times indicated. Pooled tibial bone marrows and spleens (three mice/pool) were assayed in triplicate to quantitate CFU-GM, as outlined in the *Materials and Methods* section.

response to LPS was approximately twice that observed after injection of MPL, S-TDCM, or Sm-BRM and ~15-fold above levels found in irradiated mice injected with TS. However, plasma CSF levels in LPS-treated mice declined to levels seen in irradiated controls 5 days after irradiation. Plasma CSF levels in irradiated mice injected with MPL or S-TDCM also fell rapidly but remained higher than CSF levels seen in mice injected with LPS or TS. In contrast, irradiated mice injected with Sm-BRM experienced a rise in plasma levels of CSF activity that was sustained for 24 h before declining. Measureable plasma CSF activity beyond day 7 was observed only in irradiated mice treated with Sm-BRM and S-TDCM. This late advantage in the plasma CSF response to Sm-BRM and S-TDCM is depicted in the insert in Figure 5.

Effects of BRMs on myeloid progenitors after radiation

Late myeloid progenitors (CFU-GM) in bone marrow and spleen were undetectable in all treatment groups at day 7 after irradiation (Fig. 6). However, CFU-GM appeared in both the bone marrow and spleen in all BRM treatment groups but not irradiated controls by day 14 after irradiation. CFU-GMs were not detected in bone marrow or spleen of TS-treated, irradiated mice until day 21. At day 14, splenic CFU-GM were ~2-fold greater in mice treated with S-TDCM and Sm-BRM compared with LPS and MPL treatment groups. Compared with all other treatment groups, bone marrow CFU-GM were ~1.5-fold higher in Sm-BRM-treated mice at day 21.

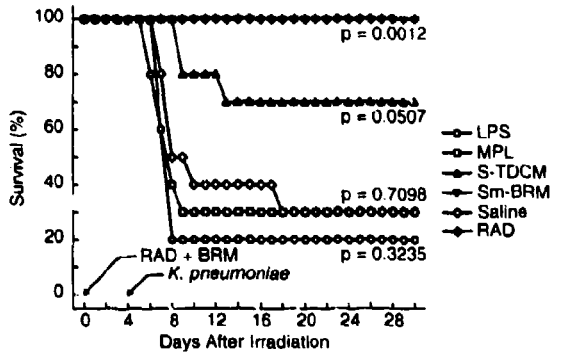

FIGURE 7. Comparative effects of BRMs on 30-day survival in sublethally gamma irradiated mice inoculated with *K. pneumoniae*. One hour after irradiation, LPS, MPL, S-TDCM, Sm-BRM, or saline was injected into groups of 10 mice and 4 days later mice were given a subcutaneous inoculation of 6.1×10^2 CFU (2 $LD_{50/30}$). Survival in each experimental group was noted daily for 30 days after irradiation.

Effects of BRMs on survival in irradiated mice challenged with K. pneumoniae

To determine whether the differences that were observed in cytokine gene expression, plasma CSF levels, or myeloid progenitor recovery among experimental groups altered survival in response to sepsis, BRMs were used in an experimental infection model in irradiated mice. One hour after irradiation, groups of 10 mice received a single i.p. injection of a BRM or TS. Four days later, mice received a s.c. inoculation of either saline or 6.1×10^2 CFU (2 $LD_{50/30}$) of a washed suspension of *K. pneumoniae*. Survival was assessed for 30 days and *K. pneumoniae* was isolated from heart blood of dead mice to confirm the cause of infection. The resulting survival curves, depicted in Figure 7, revealed that all mice treated with Sm-BRM survived. Mice that were treated with S-TDCM suffered no mortality until the 5th day after bacterial challenge, and the 30-day survival was 70%. In contrast, ≤30% of irradiated mice that were injected with saline, LPS, or MPL survived infection. The majority of mortalities in these experimental groups occurred within 7 days of bacterial challenge.

Discussion

Our data demonstrated a rapid, transient increase in endogenous splenic gene expression of IL-1β, IL-3, IL-6, and G-CSF in response to sublethal irradiation. The cytokine gene response observed in splenic tissue in our in vivo model appeared to be specific because gene expression of TNF-α, SCF, and M-CSF was not amplified after irradiation. The data also indicated that expression of cytokine genes could be differentially enhanced and prolonged, depending on the BRM used. Although LPS and MPL elicited abbreviated splenic cytokine gene responses and transient increases in plasma CSF activity, both S-TDCM and Sm-BRM elicited more prolonged gene responses and an early and late increase in plasma CSF activity. In addition, prolonged expression of multiple cytokine genes, such as that seen after a single injection of S-TDCM or Sm-BRM, was associated with superior protection against experimental infection

No previous in vivo studies to our knowledge have provided direct evidence of endogenous cytokine gene expression after irradiation but several in vivo studies have provided indirect evidence that reconstitution of medullary and extramedullary hematopoiesis after sublethal irradiation is mediated by hematoregulatory cytokines (3, 4, 6, 8, 36, 37). Neta and Oppenheim (6) reported that a single i.p. injection of IL-1 1 to 3 h after lethal irradiation improved survival in a dose-dependent fashion in mice, presumably due to known myelopoietic effects of IL-1 (38–40). Administration of IL-3, IL-6, G-CSF, and GM-CSF has also hastened myelorestoration after irradiation by enhancing proliferation of myeloid progenitors (6, 41–45).

Irradiation did not induce increased expression of TNF-α, SCF, and M-CSF genes. The absence of splenic expression of these cytokines may be a reflection of the specificity of the cytokine gene response to sublethal irradiation. Alternatively, an apparent lack of expression of these genes may have resulted from tight gene regulation and by waiting 5 h after irradiation to sample spleens transient early gene expression may have been missed. Evidence for this possibility has been found in cell culture studies in which ionizing radiation induced transient gene expression of TNF-α and IL-1 that returned to baseline within 6 h (46, 47). Several in vivo studies have provided indirect evidence that endogenous expression of TNF-α, SCF, and M-CSF is induced after irradiation and is important for survival. Therapeutic administration of TNF-α improved survival in lethally irradiated mice and injection of an Ab that is specific for TNF-α before irradiation decreased survival (6, 12). Chronic administration of recombinant canine SCF restored bone marrow hematopoiesis and enhanced recovery of circulating neutrophils in lethally irradiated dogs (10) and injection of an Ab specific for SCF before radiation decreased survival in mice (14). Similar studies with Abs specific for M-CSF have not been reported, but administration of M-CSF accelerated hematopoietic regeneration after bone marrow ablation in mice (48). Therefore, it is possible that expression of genes for TNF-α, SCF, and M-CSF occurred after irradiation in our murine model but probably took place in extrasplenic tissues, such as endothelium, bone marrow stroma, or skin (16, 49–51).

The mechanisms responsible for prolonged cytokine gene expression in sublethally irradiated mice injected with S-TDCM or Sm-BRM are unknown but the manner in which macrophages ingest and subsequently degrade S-TDCM and Sm-BRM may explain the protracted cytokine gene response (31, 52, 53). The superior ability of

Sm-BRM to protect septic mice may represent synergistic effects of LPS and non-LPS-related components of Sm-BRM. Ribi et al. (54) showed that a combination of MPL and S-TDCM was superior to either BRM alone in improving survival to *Salmonella enteritidis* infection. It is possible that Sm-BRM, by providing both LPS and non-LPS components of the bacterial cell wall, ensured a similar synergistic advantage in our study.

The ranked order of the ability of BRMs to induce and sustain cytokine gene expression (Sm-BRM > S-TDCM > LPS ≈ MPL ≈ TS) paralleled the ability of each group to survive infection. This relationship was most pronounced for IL-6 gene expression. IL-6 has been shown to improve survival after a septic challenge by decreasing overexpression of IL-1 and TNF-α, increasing a protective hepatic acute phase protein response, and augmenting adrenocortical responsiveness (13, 49–51). In addition, all BRMs induced an early plasma CSF response and an accelerated appearance of CFU-GM in bone marrow and spleen. Enhanced gene expression of IL-3 was observed in Sm-BRM-treated, irradiated mice and may have been partly responsible for the late increase in plasma CSF levels and improved survival to infection. IL-3 synergizes with M-CSF in vitro to enhance myeloproliferation and prevents apoptosis by reducing the rate of DNA cleavage during a G_2 arrest point, which occurs in the first few hours after radiation (55, 56).

Although BRMs may have been present in plasma samples that were assayed for CSF activity, it is unlikely that a carryover of BRMs was responsible for the measured CSF activity. In our hands, spiking plasma samples with BRMs before bioassay does not increase colony formation (i.e., CSF activity). Comparable plasma CSF responses have been noted by other investigators in normal and irradiated mice injected with LPS (16, 31, 57, 58). The importance of this prolonged, endogenous expression of CSFs in mice injected with S-TDCM and Sm-BRM in this study is suggested by earlier studies that reported that chronic administration of hematoregulatory cytokines accelerated bone marrow recovery and improved survival after myeloablative therapy (8, 9, 43, 59–61).

Bone marrow and splenic proliferation of CFU-GM were absent in all treatment groups at day 7 after irradiation, and no correlation between the absolute number of CFU-GM and postinfection survival at day 7 was evident. Other studies have documented the absence of myeloid progenitors and circulating neutrophils 7 days after irradiation, even after therapy with hematopoietic cytokines (8, 9, 43). The lack of correlation between the number of myeloid progenitors found in the bone marrow and host survival after irradiation has been noted by others (17, 55, 58, 62).

On balance it appears that sublethal irradiation induces limited splenic cytokine gene expression that can be expanded, amplified, and prolonged by a single i.p. injection of BRM 1 h after irradiation. Kinetics of the BRM-associated cytokine gene response to radiation depends on the particular BRM used. LPS and MPL elicit an abbreviated cytokine gene expression and initiate prompt but transient increases in plasma CSF activity. S-TDCM and Sm-BRM elicit similar early responses but the responses tend to be more prolonged. The ability of irradiated mice to withstand a septic challenge appears to depend on the amplitude and duration of cytokine gene expression and the genes that are expressed. Induction of a broad-based cytokine response after sublethal irradiation, such as that seen after a single injection of S-TDCM or Sm-BRM, is associated with the best protection against septic mortality. Additional studies need to be performed to determine whether these BRMs are efficacious in the setting of lethal gamma irradiation, with and without superimposed infection.

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